Abstract:

Bisphosphonates and Hormone Replacement Therapy (HRT) have been extensively utilized as the primary therapeutic interventions for Postmenopausal Osteoporosis (PMO) over several decades. However, the repercussions associated with their chronic usage pose a serious clinical challenge and underscore the need for innovative therapies. To address this challenge, herein we hypothesized the codelivery of hydroxyapatite (HAP) with daidzein for synergistic treatment of PMO. Propounding this bimodal approach, hydroxyapatite-nanoparticles (HAPNPs) laden with daidzein (DZ) were prepared leveraging the estrogenic properties of DZ while utilizing HAP to facilitate biomineralization. In the very first step, hydroxyapatite nanoparticles (HAPNPs) were synthesized using the chemical-precipitation method followed by drug loading (DZHAPNPs) via sorption. Fabrication and optimization of both blank and drug loaded nanoparticles was done on basis of QbD approach for minimal particle size, optimum zeta-potential, maximum drug loading and entrapment. The optimized blank nanoparticle synthesized were comprised of calcium and phosphate precursors in molar ratio of 1:0.6, and nanoprecipitation occurred at pH above 10. For maximum drug loading, the best results were found in the formulation having drug and blank nanoparticles in the ratio of 1:2. The particle size of HAPNPs and DZHAPNPs were found to be 118.9±0.15nm and 129.3±0.65nm, respectively, which were in very much agreement with their FESEM and TEM images. A notable entrapment efficiency of 87.23±0.97 % and cumulative percent drug release of 91±0.85% from DZHAPNPs was observed over 90 hours at pH 7.4. Furthermore, the optimized nanoparticles and underwent various *in vitro* and *in vivo* characterizations. In XRD results, amorphization of HAPNPs upon drug loading was confirmed in terms of their reduced crystallinity from 88% to 56 %. However, TGA analysis indicated the thermal stability of both blank and drug loaded nanoparticles up to 900^0C.

The osteogenic potential of developed nanoparticles was validated through *in-vitro* experiments on MG-63 osteoblast cells The drug-loaded nanoparticles (DZHAPNPs) exhibited pronounced pro-osteogenic activity, as evidenced by enhanced (155.49%) Alkaline Phosphatase (ALP) activity in MG-63 cells. Additionally, cellular uptake studies confirmed the effective internalization and targeted delivery of DZHAPNPs.

Furthermore, *in vivo* pharmacokinetic and pharmacodynamic studies were conducted in rodent models to confirm and potentiate the results of invitro cell line studies. Upon pharmacokinetic investigation on female Wistar rats, a three-fold increased bioavailability of DZHAPNPs ($AUC_{0-\infty}$=7427.6 µg/ml*h) was obtained in comparison to daidzein solution ($AUC_{0-\infty}$ = 2299.7 µg/ml*h).

To mimic natural menopause, a "4-vinyl cyclohexene diepoxide (VCD)-induced menopausal model" in albino mice was developed. Following successful menopause induction and treatment, the mice underwent radiography, histology, micro-CT analysis, and biochemical evaluations. The biomarkers, tartrate-resistant acid phosphatase (TRAP-5b) and serum cross-linked C-telopeptide of type I collagen (β-CTx), was significantly decreased ($p < 0.001$) after therapy, indicating a significant impact of DZ and DZHAPNPs. Better bone architectural parameters and bone mineral density in micro-computed tomography analysis served as proof of the hypothesis. The findings of micro-CT-based results and radiological studies were well corroborated with the results of histopathology of femoral bones.

Moreover, the noteworthy results of the research substantiated the synergistic influence of DZ and HAPNPs in resilience and bone strength maintenance. Additionally, the cellular-biocompatibility of developed nano-formulation was confirmed by genotoxicity tests performed on the Oregon-R strain of Drosophila melanogaster.

In conclusion, the developed phytoestrogen-biomineral-nanoparticles (DZHAPNPs) demonstrated that DZ not only ameliorated PMO but also promoted significant bone repair and regeneration due to synergism with hydroxyapatite nanoparticles and thus the nanoparticles hold promise for the treatment of PMO. These findings could improve the sensible usage of phytoestrogens in other hormone-dependent diseases and excel in their utilization.